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Pestalotiopsis lijiangensis sp. nov., a new endophytic fungus from Yunnan, China

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Abstract—A new endophytic fungus, *Pestalotiopsis lijiangensis* from leaves of *Castanopsis carlesii* var. *spinulosa*, is described and illustrated. The new species is similar to *P. montellica* and *P. jesteri* in having lateral appendages at the upper conidial septum, but *P. lijiangensis* differs from both *P. montellica* and *P. jesteri* by its wider conidia with deeply pigmented doliform median cells and additionally from *P. jesteri* by the production of apical conidial appendages. A phylogenetic analysis of the combined sequence data of internal transcribed spacer (ITS), partial β-tubulin (tub), and partial translation elongation factor1-α (tef) supports *P. lijiangensis* on an independent branch within *Pestalotiopsis*.

Key words—*Amphisphaeriales,* coelomycetes, molecular phylogenetics, *Pestalotiopsidaceae*, taxonomy

Introduction

Pestalotiopsis Steyaert is characterized by relatively fusiform conidia, each comprising a hyaline basal cell, three pigmented median cells, and a hyaline apical cell with two or more apical appendages (Steyaert 1949). The interspecific delineation of *Pestalotiopsis* is based on conidial morphology (Guba 1961, Nag Raj 1993), conidiogenesis (Sutton 1980), and teleomorph association (Barr 1975, 1990; Zhu & al. 1991; Metz & al. 2000). The use of molecular data in resolving *Pestalotiopsis* species has been reviewed by Hu & al. (2007), Tejesvi & al. (2007), Liu & al. (2010), and Maharachchikumbura & al. (2011). These studies suggest that multi-locus phylogenetic analysis is needed to resolve the

cryptic species in the genus. A combination of internal transcribed spacer (ITS), partial β-tubulin (TUB), and partial translation elongation factor1-α (TEF) gene data gave the best resolution when compared to single gene analysis (Maharachchikumbura & al. 2012). Based on these analyses, *Pestalotiopsis* sensu lato has been divided into three genera: *Pestalotiopsis* sensu stricto, *Neopestalotiopsis* Maharachch. & al., and *Pseudopestalotiopsis* Maharachch. & al., after morphology and sequence data (ITS, TUB, and TEF) were combined to identify new cryptic species (Maharachchikumbura & al. 2014).

Here, we isolated two endophytic fungal strains from a leaf of *Castanopsis carlesii* var. *spinulosa* W.C. Cheng & C.S. Chao (*Fagaceae*); both strains shared the character of lateral appendages at the upper conidial septum with *P. montellica* (Sacc. & Voglino) Tak. Kobay. and *P. jesteri* Strobel & al. (nom. inval.) but differed from those species by the size and pigmentation of their conidia. We therefore propose this endophytic fungus as a new species, *Pestalotiopsis lijiangensis*. Its phylogenetic position was inferred from the combined sequences of ITS, TUB, and TEF.

Materials & methods

Observation of morphological characters

Conidia were produced in a monoculture derived from a single conidium that was selected for morphological observation. Morphological characters for identification were measured and photographed under an Olympus Bx51 light microscope. These characters included conidial length and width, appendage length and number, and the pigmentation of the three median conidial cells. Microscopic examination performed 30 repetitions.

DNA extraction, amplification, sequencing

Total DNA was extracted from fresh cultures following the protocol of Guo & al. (2000). For nucleotide sequence comparisons, nrLSU, ITS, TUB and TEF genes were amplified using primer pairs LR0R/LR5 (Vilgalys & Hester 1990, Rehner & Samuels 1994), ITS5/ITS4 (White & al. 1990), T1/Bt-2b (Glass & Donaldson 1995, O'Donnell & Cigelnik 1997), and EF1-728F/EF-2 (O'Donnell & al. 1998, Carbone & Kohn 1999), respectively. Amplification conditions for nrLSU, ITS and TEF followed Crous & al. (2012) and for TUB followed Lee & al. (2004). The PCR products were purified and edited by Zhongkexilin Biotechnology Co. Ltd. (Beijing, China). The new sequences were submitted to the GenBank database. Other sequences in this study were downloaded from GenBank (TABLE 1).

Phylogenetic analyses

The sequences were aligned with Clustal X1.81 (Thompson & al. 1997) and the results were adjusted manually where necessary to maximize alignment. All sites

Table 1. *Neopestalotiopsis* and *Pestalotiopsis* sequences used in the molecular analysis

Species & voucher	GenBank Accession No.		
	ITS	TUB	TEF
N. saprophytica MFLUCC12-0282	JX398982	JX399017	JX399048
P. camelliae CBS443.62	KM199336	KM199424	KM199512
P. clavata MFLUCC12-0268	JX398990	JX399025	JX39905
P. cocculi HHL-BZ	KM535703	KM573239	—
P. colombiensis CBS118553	KM199307	KM199421	KM199488
P. disseminata EC3A	EF055196	EF055233	—
P. diversiseta MFLUCC12-0287	JX399009	JX399040	JX399073
P. gracilis QQ-OZ	HM573281	HM535746	—
P. hawaiiensis CBS114491	KM199339	KM199428	KM199514
P. intermedia MFLUCC12-0259	JX398993	JX399028	JX399059
P. jesteri CBS109350	KM199380	KM199468	KM199554
"*P. jesteri*" MFLUCC12-0279	JX399012	JX399043	JX399076
P. knightiae CBS114138	KM199310	KM199411	KM199482
P. lijiangensis CFCC50738 [T]	KU860520	KU844184	KU844185
P. lijiangensis CFCC50739	MH8808834	MH880835	MH880836
P. malayana CBS102220	KM199306	KM199411	KM199482
P. microspora P20-02	HQ315843	—	—
P. neglecta QQ-TKZ	HM535758	HM573293	—
P. olivacea SY17A	EF055215	EF055251	—
P. portugallica CBS393.48	KM199335	KM199463	—
P. rosea MFLUCC12-0258	JX399005	JX399036	JX399069
P. verruculosa MFLUCC12-0274	JX398996	—	JX399061
P. vismiae HHL-DG	HM535704	HM573246	—

were treated as unordered and unweighted, and gaps were treated as missing data in the phylogenetic analyses. Firstly, new species in *Pestalotiopsis* sensu stricto, *Neopestalotiopsis*, and *Pseudopestalotiopsis* was estimated by the nrLSU sequence data. The result grouped *P. lijiangensis* in *Pestalotiopsis* sensu stricto (not shown). Analysis of a combined alignment dataset (ITS, TUB, and TEF) confirmed the relationship of the new species and other congeneric species in *Pestalotiopsis*. The alignment data was subsequently used for maximum parsimony (MP) analysis, in which a search for most parsimonious trees was conducted with the heuristic search algorithm with tree-bisection-reconnection (TBR) branch swapping in PAUP 4.0b10 (Swofford 2003). For each search, 1000 replicates of random stepwise sequence addition were performed, and all trees were saved per-replicate. The strength of the internal branches of the trees was tested with bootstrap analyses

using 1000 replications with the same search settings. For the Bayesian analysis, MrModeltest 3.7 with the Akaike information criterion was used to choose the substitution model for the separate dataset (Nylander & al. 2004), and the analysis was performed with MrBayes 3.1.2 (Huelsenbeck & Ronquist 2001, Ronquist & Huelsenbeck 2003). The detailed methods and parameters of MP and Bayesian analyses followed Maharachchikumbura & al. (2014). Bayesian posterior probabilities (PP) were obtained from the 70% majority rule consensus of the trees kept. Bootstrap percentages (BP) of more than 70 from 1000 replications are shown above the respective branches (Fig. 2). If >95% of the sampled trees were contained in a given clade, it was considered to be significantly supported by the data.

Taxonomy

Pestalotiopsis lijiangensis Y.K. Zhou & C.L. Hou, **sp. nov.** Fig. 1

MycoBank MB 816081

Differs from *Pestalotiopsis montellica* by its wider conidia with deeply pigmented doliform median cells; and from *P. jesteri* by its wider conidia with deeply pigmented doliform median cells and by producing apical conidial appendages.

Type: China, Yunnan, Lijiang, alt. ca. 3800 m, endophytic in living leaves of *Castanopsis carlesii* var. *spinulosa*, 18 June 2014, Y.K Zhou. **Holotype,** BJTC 1181; ex-type culture, CFCC50738; GenBank KU860520, KU844184, KU844185.

Etymology: *lijiangensis* referring to the city where this species was obtained.

Conidiomata pycnidia in culture on PDA, globose, scattered, semi-immersed, black, <100–300 µm diam; exuding globose, dark brown to black conidial masses. Conidiophores sparsely septate at base, branched or unbranched, subcylindrical, hyaline, smooth, <15 µm or reduced to conidiogenous cells. Conidiogenous cells discrete, lageniform to subcylindrical, hyaline, smooth, 10–25 × 2–5 µm (x = 13.5 × 3.7 µm), proliferating 1–3 times percurrently. Conidia fusiform, 4-septate, 22–25 × 10–12 µm (x = 23.8 × 11.5 µm), mean conidial length/width ratio = 2.96; bearing appendages; basal cell narrowly obconic with a truncate base bearing minute marginal frills, hyaline, smooth, 2–4 µm (x = 3.4 µm); three median cells doliform, brown, concolourous, walls verruculose and slightly constricted at the septa, together 14.5–18 µm (x = 16 µm), second cell from base 4.5–5.5 µm (x = 5.3 µm); third cell 4–5.1 µm (x = 4.4 µm); fourth cell 6–7.4 µm (x = 6.3 µm); apical cell obconic with an acute apex, hyaline, smooth, 2–3.5 µm (x = 2.8 µm). Appendages tubular, unbranched, flexuous, more or less attenuated; apical appendage single, 3–8 µm (x = 6.7 µm); lateral appendages 2–4 (mostly 3), arising just above the septum separating the apical cell and subapical cell, unbranched 14–25 µm (x = 18.6 µm); basal appendage (occasionally absent) unbranched, attenuated, flexuous, centric 2–6 µm (x = 4.8 µm).

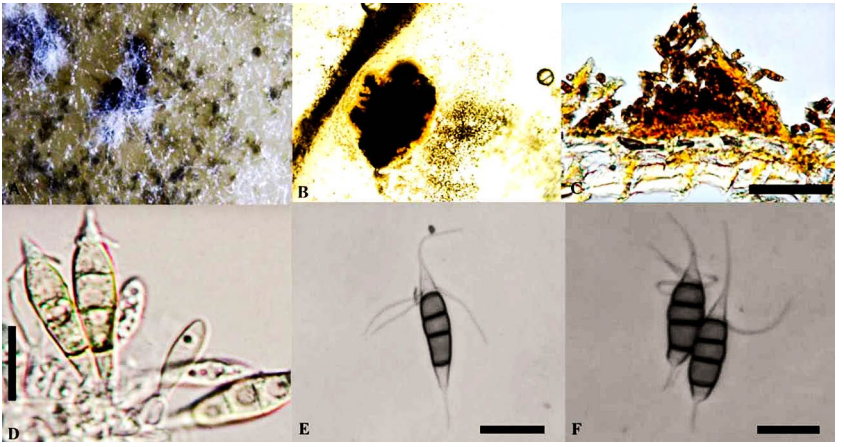


Fig. 1 *Pestalotiopsis lijiangensis* (CFCC50738): A. Conidiomata on PDA; B. Conidioma sporulating on PDA; C. Section of conidioma, split irregularly; D. Conidiophores and immature conidia; E, F. Conidia. Scale bars: C = 50 µm; D = 30 µm; E, F = 20 µm.

Additional material examined: **CHINA, Yunnan, Lijiang,** alt. ca. 3800 m, endophytic in living leaves of *Castanopsis carlesii* var. *spinulosa*, 18 June 2014, Y.K. Zhou (BJTC 1182, CFCC 50739; GenBank MH880834, MH880835, MH880836).

Phylogeny

To clarify species boundaries within *Pestalotiopsis*, a combined alignment of ITS, tub, and tef was created, containing 23 sequences (including the outgroup *Neopestalotiopsis saprophytica*) and 1519 characters. Of the 1519 characters (ITS = 552, tub = 463, and tef = 504), 890 were constant, 250 variable characters parsimony uninformative, and 379 characters parsimony informative. The MP analysis of sequences resulted in one most parsimonious tree with a length (TL) of 1738 steps, consistency index (CI) of 0.636, retention index (RI) of 0.708, homoplasy index (HI) of 0.604, and rescaled consistency index (RC) of 0.587. The Bayesian analysis resulted in a tree with the same topology and terminal clades as MP trees. *Pestalotiopsis* sensu stricto formed two well supported branches, and the new species *Pestalotiopsis lijiangensis* (CFCC 50738, CFCC 50739) was in a strongly supported independent clade (BP = 100, PP = 1) (Fig. 2).

Discussion

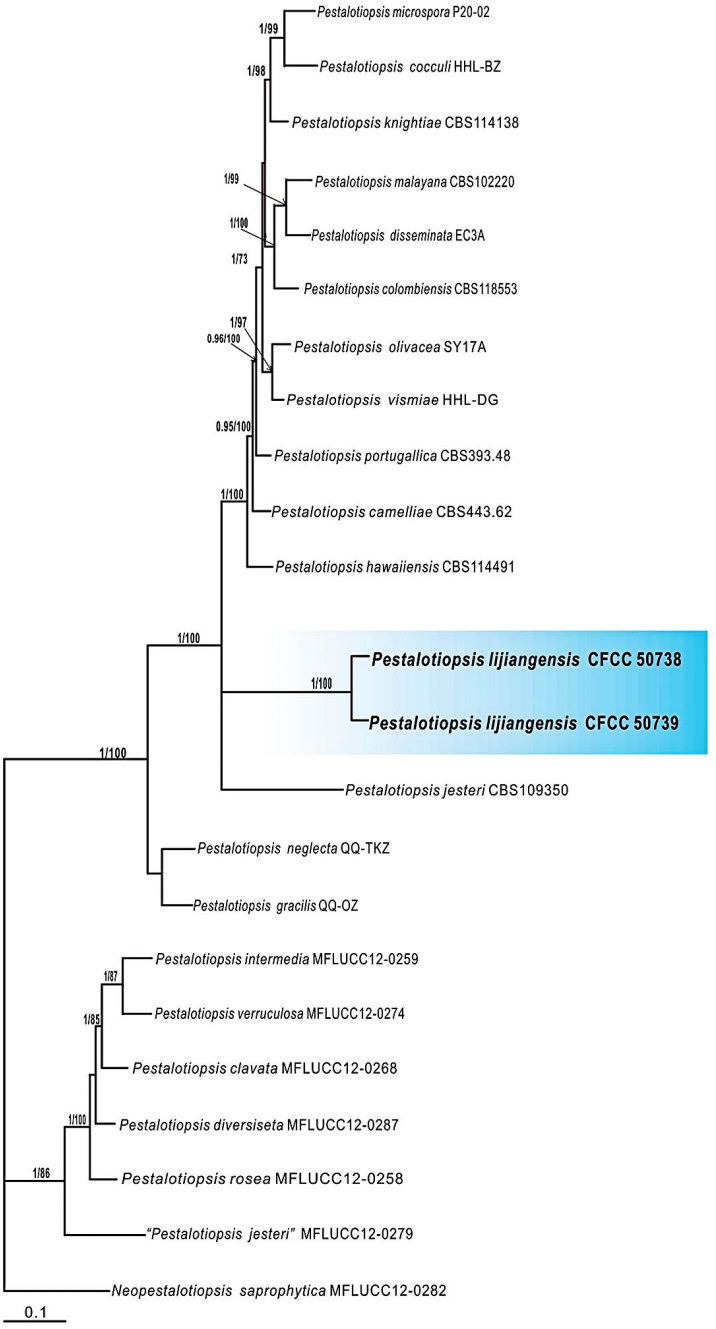
Conidial morphology is the most widely used taxonomic character for species in *Pestalotiopsis*, most species are identified by conidial size (Maharachchikumbura & al. 2011).

As can be seen from Table 2, the difference between *P. lijiangensis* and *P. montellica* is mainly the conidial length/width ratio, with the ratio of *P. montellica* almost 1.5 times that of *P. lijiangensis*. Furthermore, the lateral appendages of *P. montellica* are significantly shorter than those of *P. lijiangensis*. Although the conidial length/width ratios of *P. lijiangensis* and *P. jesteri* are similar, the three median cells are remarkably different: in *P. jesteri* the center cell is slightly smaller than the two others, while in *P. jesteri* the center cell is bigger (Strobel & al. 2000). Besides, the conidium of *P. jesteri* has no apical appendages, while *P. lijiangensis* has an unbranched apical appendage. Molecular analyses of the combined datasets cluster *P. lijiangensis* as an outlying species with high branch-length support. Unfortunately, no DNA sequence of *P. montellica* is available, so further research is needed to determine the relationship between *P. lijiangensis* and *P. montellica*.

Table 2. Morphological comparison of *Pestalotiopsis lijiangensis* and two similar species

Characters	Species		
	P. lijiangensis (This paper)	*P. montellica* (Guba 1961)	*P. jesteri* (Strobel & al. 2000)
Conidium morphology			
Length (µm)	18.5–27, x = 22.5	22–32, x = 26.5	19–23, x = 19
Width (µm)	6–9, x = 7.5	5–7, x = 5.7	5–7, x = 6
Mean length/width ratio	2.96	4.6	3.2
Median cell shape/color	Doliform/brown	Cylindrical/pale brown	Fusiform/pale brown
Lower median cell (µm)	4.5–6, x = 5.3	6–7.5, x = 6.7	4.8–5.2, x = 5
Center median cell (µm)	4–5.1, x = 4.4	3–5.5, x = 4.6	5.3–5.8, x = 5.5
Upper median cell (µm)	6–7.4, x = 6.3	4–6.5, x = 5.2	4.8–5.1, x = 4.9
Apical appendages			
Number	1	1	0
Branching	unbranched	unbranched	—
Length (µm)	2–4, x = 3.4	5–7, x = 6	—
Lateral appendages			
Number	(2–)3(–4)	(2–)3(–4)	3
Length (µm)	14–25, x = 18.6	4–12, x = 8	11–28, x = 23
Basal appendages			
Number	(0–)1	0(–1)	1
Branching	Unbranched	Unbranched	Unbranched
Length (µm)	2–6, x = 4.8	2–6, x = 4	3–8, x = 4.5

Fig. 2 Phylogenetic tree derived from maximum parsimony analysis of the combined (ITS+tub+tef) sequences of *Pestalotiopsis* spp., with *Neopestalotiopsis. saprophytica* as outgroup. Branch support is shown as: Bayesian posterior probabilities >0.95 / bootstrap values >70%.


Pestalotiopsis microspora P20-02
1/99
Pestalotiopsis cocculi HHL-BZ
1/98
Pestalotiopsis knightiae CBS114138
1/99
Pestalotiopsis malayana CBS102220
1/100
Pestalotiopsis disseminata EC3A
1/73
Pestalotiopsis colombiensis CBS118553
1/97
0.96/100
Pestalotiopsis olivacea SY17A
Pestalotiopsis vismiae HHL-DG
0.95/100
Pestalotiopsis portugallica CBS393.48
1/100
Pestalotiopsis camelliae CBS443.62
Pestalotiopsis hawaiiensis CBS114491
1/100
Pestalotiopsis lijiangensis CFCC 50738
1/100
Pestalotiopsis lijiangensis CFCC 50739
1/100
Pestalotiopsis jesteri CBS109350
Pestalotiopsis neglecta QQ-TKZ
Pestalotiopsis gracilis QQ-OZ
Pestalotiopsis intermedia MFLUCC12-0259
1/87
Pestalotiopsis verruculosa MFLUCC12-0274
1/85
Pestalotiopsis clavata MFLUCC12-0268
1/100
Pestalotiopsis diversiseta MFLUCC12-0287
1/86
Pestalotiopsis rosea MFLUCC12-0258
"Pestalotiopsis jesteri" MFLUCC12-0279
Neopestalotiopsis saprophytica MFLUCC12-0282
0.1

The two sequenced strains of *P. jesteri* appeared in different clades: CBS 109350 (a type strain from Papua New Guinea) was closely related to *P. lijiangensis,* while MFLUCC 12-0279 (from Yunnan, China) was only distantly related. The Yunnan strain was described with a conidial length/width ratio ≈ 4.1 and sequenced by Maharachchikumbura & al. (2012), who accepted it within *P. jesteri*. The Papua New Guinea strain, originally described by Strobel & al. (2000) and redescribed and sequenced by Maharachchikumbura & al. (2014), has a conidial length/width ratio ≈ 3.2. The Yunnan strain was excluded from *P. jesteri* by Maharachchikumbura & al. (2014), who included only the original Papua New Guinea material in their circumscription of the species. Further study is needed to identify the misdetermined Yunnan strain MFLUCC 12-0279.

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